Research Article

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Some genomic features of *Enterococcus durans* isolated from fermented milk products

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ABSTRACT

Enterococci are widely used in the production of various fermented food products and can be applied as probiotics. However enterococci are typical opportunistic pathogens that cause nosocomial infections, they are involved in distribution of antibiotic resistance and process of food spoilage. In the present study genome of 9 *Enterococcus durans* strains isolated from fermented milk products was analyzed in order to study intraspecies variability and identify pathogenicity, antibiotic resistance genes as well as genes encoding some enterocins. A high level of intraspecies polymorphisms was revealed as the result of amplification with primer to tetranucleotide repeat: each sample had unique amplicon pattern. On the contrary, no detectable differences between samples were observed using M13 primer which contains minisatellite repeat. *E.durans* strains were also genetically characterized to identify genes coding enterocins A, B, L50B and P. Among all only enterocin P gene was revealed in all samples. To assess the pathogenicity of isolated *E. durans* strains PCR with primers to virulence determinants, gelatinase *gelE*, cytolysin *cylA*, hyaluronidase *hyl*, adhesion factors *asa1* and *esp,* was performed. None of these was detected in strains analyzed. PCR screening for antibiotic resistance genes (*aph, ant, tetM, vanA, vanB*) showed that all strains had vancomycin and tetracycline resistance genes and 20% of strains possessed aminoglycoside resistance gene. Thus, the results obtained indicate that accurate molecular-genetic analysis of dairy products is required to prevent harmful effects on human health.

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1. INTRODUCTION

Enterococci are gram-positive bacteria that belong to lactic acid bacteria group and constitute to common human and animal intestinal microbiota. These microorganisms are widespread due to their tolerance to high temperature and ability to survive under adverse environmental conditions: they are found in food, vegetable and plant materials [1].

Enterococci play an important role in the dairy industry, taking part in the development of organoleptic properties during ripening of some cheeses [2]. These bacteria are widely used in the production of various fermented food products and can be components of starter cultures and probiotics, they also possess useful biotechnological traits. However, enterococci are typical opportunistic pathogens that cause nosocomial infections such as endocarditis, bacteremia, urinary tract infections or neonatal sepsis [3], they are involved in distribution of antibiotic resistance and process of food spoilage [4]. Considering such diverse and ambiguity role of enterococci in our life it is very important to establish accurate evaluation of *Enterococcus* species and strains and exclude potentially harmful representatives. Molecular-genetic methods and techniques are applied for this purpose including PCR with specific primers to identify bacteria and detect the presence of certain genes involved in various processes.

In our research we analyzed genome of 9 *E. durans* strains isolated from fermented milk products in order to study intraspecies variability and identify antibiotic resistance, pathogenicity genes as well as genes encoding some enterocins.

2. MATERIAL AND METHODS

2.1 Sample collection and enterococci isolation

Three samples of home-made milk product (cottage cheese) were collected from local producers in Kyiv region, Ukraine. Samples were stored in sterile bottles at 4^0C.

Samples (10 g) were diluted in 90 ml of 1% sterile saline solution and homogenized. The homogenates were serially diluted in a sterile peptone-saline solution (1 g/l peptone and 8,5 g/l NaCl). Aliquots of each dilution were streaked on MRS agar and incubated at 37^0C for 48 – 72 h under aerobic condition. For each sampling point, one to 5 colonies were randomly picked from the countable MRS agar plates and streaked out three times on the same media used for the isolation to check for purity. Colony morphology, Gram and catalase reactions were checked.

2.2 Genetic analysis

To perform genetic analysis one colony from each plate was selected and cultivated in liquid MRS media at 37 ^{o}C overnight. Total DNA was isolated from cell suspension using DNA isolation kit (“DNA-sorb B”, Russia) according to manufacturer’s instruction. *E. durans* CCM 596^T was used as reference in the genetic assays.

The identification of enterococci was carried out by PCR with species-specific primers as described in [5]. Amplification with primers to genes encoding enterocins (*entA, entB, entP, entL50B),* virulence determinants (*asa1, cylA, gelE, esp, hyl*) and antibiotic resistance (*aph, ant, tetM, vanA, vanB*) was performed according to procedures in [3,4,6–10]. To analyze intraspecies variability primer to tetranucleotide repeat 5’-$(GACA)_4$-3’ and M13 primer (5’-GAGGGTGGCGGTTCT-3’) were used in PCR; components of reaction mix, amplification procedure and analysis of amplicon patterns were published in [11].

3. RESULTS AND DISCUSSION

Lactic acid bacteria strains isolated from home-made dairy products are potential source for using them in starter cultures or as probiotics due to their unique features. In the present study *E.durans* strains isolated from home-made cottage cheese were analyzed to assess some peculiarities of bacterial pan-genome. Detection of dispensable genes that contribute to species diversity and confer selective advantages as well as analysis of nucleotide repeats distribution in *E.durans* genome were performed.

3.1 Identification and intraspecies variability of enterococci

Results of amplification with species-specific primers showed that among 28 gram-positive, catalase-negative bacteria isolated in our study 9 were identified as *E.durans* and used for further molecular-genetic analysis. *E.durans* strains have been also defined in other dairy products such as various artisanal cheeses and Turkish tulum cheese [12,13].

Bacterial genomes contain large amount of repetitive DNA in coding and non-coding regions [14]. Nucleotide repeats differ by their size, length, orientation to each other and specific nucleotide composition [15]. Recently it has become possible to use a variety of nucleotide repeats as DNA-markers to study intra- and interspecies bacterial heterogeneity due to the development of new methods and approaches of molecular-genetic analysis. In most researches they have been used to identified and distinguish microorganisms, study role of short nucleotide repeats in adaptive process and molecular mechanisms of pathogenicity, to analyze effects of mutagenic factors on genome [14,16,17]. To analyze intraspecies variability primers to the nucleotide repeats, mini- and microsatellite DNA, were used. The pattern of the fragments amplified with primer M13, containing minisatellite repeat [18], comprised 5 PCR-products with size range from 500 to 2000 bp. There were no polymorphic amplicons observed between strains (Fig. 1, A).

Analysis of PCR-fragments spectrum obtained with primer to tetranucleotide repeat revealed that each strain was characterized by its unique set of fragments (Fig. 1, B). The amount of bands for each sample counted from 6 to 11 and their size varied from 200 to 900 bp. It should be noted that the number of amplicons in the spectrum of most strains analyzed (7 among 9) was greater than in the spectrum of type strain. According to the data reported in some researches, short bacterial repeats are a part of transposable elements that are involved in the processes of recombination and horizontal gene transfer [15]. It is possible that the increasing the number of repetitive DNA sites in *E.durans* genome, observed in our study, caused by the long-term cultivation of these organisms *in vivo* and, as a result, re-organization of nucleotide sequences. Thus, results of PCR-analysis revealed that short nucleotide repeats can be used to distinguish *E. durans* strains and analyze intraspecies variability but the identification of strain-specific markers requires further research.

3.2 Evaluation of dispensable genes in *E.durans* genome

Studies the properties of enterococci isolated from dairy products showed that these organisms are able to synthesize a wide range of bacteriocins, substances that exhibit antimicrobial properties and are able to prevent the growth of pathogenic microorganisms in foods [19].

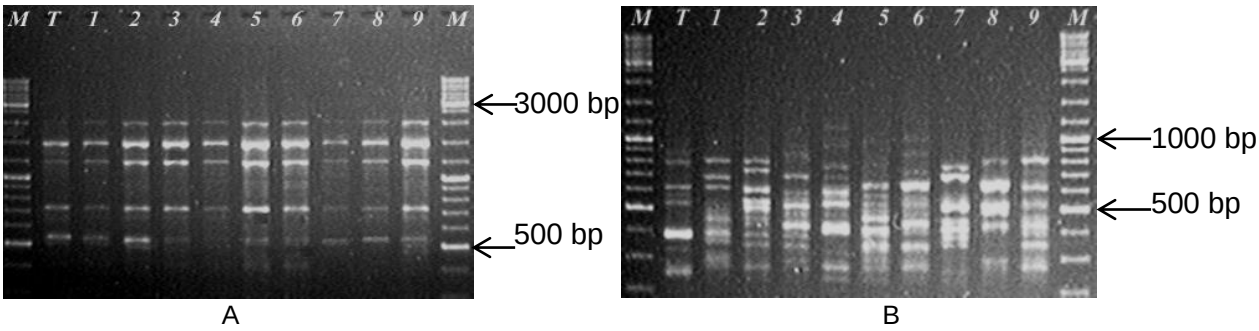

Fig. 1. Electrophoregram of fragments of amplification with primers M13 (A) and $(GACA)_4$ (B) and DNA isolated from *E.durans* strains. M – DNA-ladder mix ("Fermentas", Lithuania); T – *E. durans* CCM 596^T; 1 – 9 – *E. durans* strains

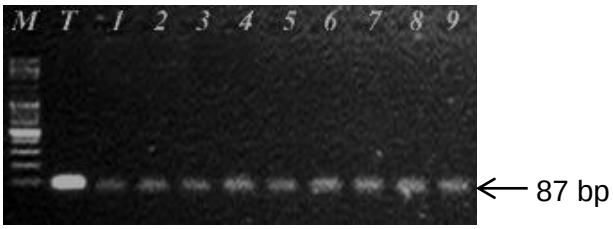

Fig. 2. Electrophoregram of PCR-amplified DNA fragment of gene encoding enterocin P. M – DNA-ladder mix ("Fermentas", Lithuania); T – *E. durans* CCM 596^T; 1 – 9 – *E. durans* strains

Results of PCR-analysis carried out in the present research revealed that *E.durans* strains genome did not contain genes encoding enterocins A, B, L50B although enterocin L50B gene was detected in type strain. Gene encoding enterocin P was observed in all samples analyzed (Fig. 2). Enterocin P was defined in most representatives of *Enterococcus* genus [20].To assess the pathogenicity of *E. durans* strains, isolated from home-made cottage cheese, genome was screened for virulence factors genes: cytolysin, gelatinase, hyaluronidase, and surface protein adhesins. None of genes encoding virulence factors was revealed in all samples analyzed.

Another important factor for the safety evaluation of enterococci is their resistance to antibiotics. Amplification with primers to antibiotic resistance genes showed that all strains possessed genes of vancomycin and tetracycline resistance, besides aminoglycoside resistance gene was observed in two strains. Resistance to different antibiotics of both natural and acquired characters has been detected among various lactic acid bacteria of different origin. A broad spectrum of antibiotic resistances within these two types has been revealed in enterococci [21]. Since antibiotic resistance genes are widely spread out in bacteria of different ecosystem [22] it is assumed that careful monitoring of these genes should be performed for safety reasons.

4. CONCLUSION

Thus, results of genetic analysis carried out in the present study revealed the high level of intraspecies variability among *E.durans* strains isolated from home-made cottage cheese; genes encoding enterocin P, resistance to vancomycin and tetracycline were defined in all strains and two strains possessed aminoglycoside resistance gene. These results indicate that accurate genetic analysis of enterococci should be performed before using them in the production of food and pharmaceutical products.

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